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THERAPEUTIC APPROACHES TO THE TREATMENT OF BOTULISM

Annual Report

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In vitro experiments have been done on isolated phrenic nerve-hemidiaphragm preparations. The purpose of the experiments was to evaluate aminopyridines as putative therapeutic agents in the treatment of botulism and to develop monoclonal antibodies that would neutralize botulinum toxin and tetanus toxin. The work demonstrates that aminopyridines are active against type A botulinum toxin. Research on antibodies is still in progress.

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1. Statement of Problem

Pharmacological methods are being sought to prevent or reverse the effects of botulinum neurotoxin. During the past year, two approaches have been tested: i.) aminopyridines have been evaluated as putative therapeutic agents for types A, B and E botulinum poisoning, and ii.) monoclonal antibodies have been sought that would neutralize both botulinum neurotoxin and tetanus toxin.

2. Background

Botulism is a neurological disorder caused by an exotoxin from the organism Clostridium botulinum. The disease is characterized by progressive muscle weakness that can result in complete flaccid paralysis. Unfortunately, there is no known cure for the disorder. Patients who contract the illness are typically provided with supportive therapy (e.g., respiratory support). Recovery appears to be a function of the ability of the nervous system to repair the damage produced by the toxin.

Botulinum toxin acts on peripheral nerve endings that store and release the transmitter acetylcholine (2,15). It shows greatest affinity for those cholinergic nerve endings that innervate striated muscle, including the muscles of respiration (e.g., diaphragm and intercostal muscle). The precise mechanism of toxin action is blockade of nerve-depolarization-induced release of acetylcholine. This action accounts for the ability of the toxin to produce muscle weakness and flaccid paralysis.

Botulinum toxin is a protein with an M_r of ~150,000 (10,15). It is composed of two polypeptide chains (M_rs, ~100,000 and

50,000) that are linked by a disulfide bond. The toxin appears to proceed through a sequence of three steps in producing its neuroparalytic effects (14,15). There is an initial binding step, a subsequent membrane penetration step, and an eventual poisoning step that occurs inside cholinergic nerve endings. The binding and internalization steps are thought to be mediated by the heavy polypeptide chain (L. L. Simpson, Ann. Rev. Pharmacol. Toxicol., in press). The intracellular poisoning step may be mediated by the light chain.

The incidence of human botulism is relatively low, and thus programs of immunization are not deemed appropriate. When individual patients contract the disease, there is the possibility of administering antitoxin, but this is of limited value. Antitoxin will neutralize circulating titers of botulinum toxin, but it cannot cross the plasma membrane of nerve cells to neutralize internalized toxin. Therefore, alternate pharmacological means have been sought to overcome the effects of botulism.

The fact that the toxin depresses transmitter release has encouraged efforts to find therapeutic drugs that will promote acetylcholine release. The aminopyridines (4-aminopyridine [4-AP] and 3,4-diaminopyridine [3,4-DAP]) are one group of agents that has been tested. These drugs act on nerve membranes to promote influx of calcium, and this in turn greatly promotes efflux of acetylcholine (4, 9, 18). The pharmacological actions of 4-AP and 3,4-DAP, as well as certain preliminary observations (7), suggest that these drugs may be useful in the treatment of

botulism. More specifically, they might i.) slow the onset of neuromuscular blockade, ii) increase the lethal dose, or iii) provide symptomatic relief.

The present study is an attempt to evaluate two of these possibilities. Data are described that help to evaluate 4-AP and 3,4-DAP as agents that can slow the onset of neuromuscular blockade or provide symptomatic relief.

Another possible avenue through which to develop therapeutic agents is to prepare synthetic antigens. Past work suggests that the synthetic antigen most likely to be effective is one that is derived from the carboxyterminus of the heavy chain, i.e., the portion of the molecule that mediates binding. This research involves a sequence of steps, as follows:

A. Isolate the binding fragment (50,000 dalton carboxy-terminus) from the botulinum neurotoxins most often implicated in human botulism (types A, B and E).

B. From the binding component, isolate the segment that is responsible for fixation to the tissue receptor. Work on other protein toxins indicates that this segment will surely be less than 10,000 daltons, and may be less than 5,000 daltons.

C. Sequence the small polypeptide domains actually involved in receptor binding.

D. Synthesize a polypeptide(s) that conserves the greatest amount of sequence homology from the various toxins.

E. Test the synthetic agent as a vaccine that elicits neutralizing antibodies.

3. Methods

Most studies were conducted on isolated phrenic nerve - hemidiaphragms that were excised from animals and placed in either a tissue bath or an incubation bath. Tissues were maintained in a physiological solution that was bubbled with 95% O_2 and 5% CO_2. The solution had the following composition (millimolar): NaCl, 137; KCl, 5; $CaCl_2$, 1.8; $MgSO_4$, 1.0; $NaHCO_3$, 24; Na_2HPO_4, 1.0; glucose, 11. Solutions were supplemented with gelative (0.02%) to diminish adsorption and nonspecific inactivation of toxins. The tissue baths were kept at 35 to 36°C, and the incubation tubes were kept at 4°C.

The parameters of phrenic nerve stimulation were 0.2-Hz square waves of 0.1 to 0.3 ms duration. Muscle twitch was recorded with a force-displacement transducer connected to a physiological recorder. Toxin-induced paralysis of neuromuscular transmission was measured as a 90% reduction in muscle twitch amplitude evoked by nerve stimulation. In keeping with the mechanism of toxin action, paralysis was irreversible.

In some experiments, the left phrenic nerve-hemidiaphragms of rats were poisoned in vivo. Animals were anesthetized with sodium pentobarbital (50mg/kg of body weight, intraperitoneally), after which they were secured to an operating table that was inclined. An incision was made approximately 6 mm above the diaphragm, and the left lobes of the lungs were displaced. A bipolar electrode was placed on the phrenic nerve, which was then stimulated at 0.2 Hz. Muscle twitch was recorded as described above. A solution of botulinum toxin was swabbed across the

hemidiaphragm, and muscle twitch was recorded until nerve stimulation failed to evoke a muscle response. When neuromuscular transmission was blocked, the wound was surgically closed. Animals received an intravenous injection of botulinum antitoxin (bivalent botulism antitoxin; Lederle Laboratories, Pearl River, N.Y.) to prevent development of generalized botulism.

4a. Results and Conclusions from Aminopyridine Experiments

4-Aminopyridine (4-AP) and 3,4 - diaminopyridine (3,4-DAP) act on ion channels in excitable membranes. The endresult of this action is a greatly promoted influx of calcium ions, which in turn triggers the release of acetylcholine (4,18). This mechanism of action suggests that aminopyridines might exert one or more effects that would be beneficial in botulism. Indeed, there is already preliminary evidence to support this idea.

A number of investigators have shown that 4-AP and 3,4-DAP can act as antagonists of botulinum neurotoxin (5, 7-9, 12, 13) and tetanus toxin (1, 3). With only one exception (7), these studies were not aimed at evaluating the therapeutic potential of aminopyridines. The goal of the studies was to determine the mechanism of action of the neurotoxins. However, in each case, data were presented that showed that aminopyridines did possess some antagonistic activity. And in the report by Lewis, Jr. (7), preliminary results indicated that aminopyridines could delay the onset of symptoms in animals poisoned with botulinum toxin.

Despite these favorable suggestions, there are two potential and serious drawbacks. First, studies done on isolated tissues

that were excised from animals previously poisoned with botulinum toxin have shown a heterogeneity of effect. The aminopyridines were not equivalently effective in relieving the neuroparalytic symptoms produced by different types of botulinum toxin (5, 12). Second, there is a theoretical concern that has diminished enthusiasm for pursuing studies on the aminopyridines. It has been repeatedly demonstrated that the rate of onset of toxin-induced neuromuscular blockade is nerve activity dependent; i.e., the more rapid the rate of nerve stimulation, the more rapid the onset of paralysis. This may be due to the link between exocytosis (viz., the release of acetylcholine) and endocytosis (viz., the internalization of toxin). This allows the possibility that aminopyridines could exert opposing actions. On one hand, they would antagonize poisoning by promoting acetylcholine release; on the other hand, they would enhance toxicity by promoting uptake of toxin molecules.

The present study provides an evaluation of aminopyridines as therapeutic agents in botulism. Experiments were performed that bear directly on two issues, the ability of the drugs to slow the onset of paralysis and the ability of the drugs to provide symptomatic relief. Of the seven serotypes of botulinum neurotoxin, three were examined (A, B, and E). It is these three that account for most cases of human botulism.

When tested at the maximum concentration practical for study (10^{-4}M), both 4-AP and 3,4-DAP slowed the rate of onset of neuromuscular blockade produced by botulinum toxin type A. Of the two, 3,4-DAP produced a more striking effect. Interestingly,

neither drug was equally effective in delaying the onset of paralysis due to botulinum toxin types B and E or tetanus toxin.

Failure of the drugs to provide substantial protection against botulinum toxin types B and E cannot be due to drug-promoted uptake of the toxins. When the paralysis times of control tissues and toxin-treated tissues were compared, it was found that the aminopyridines did not cause tissues to begin to be paralyzed more quickly. Furthermore, the aminopyridines did not provide substantial protection against botulinum toxin types B and E when added to tissues that had already internalized the toxin.

When tested for its ability to relieve the effects of poisoning in tissues that had previously been exposed to botulinum toxin, 3,4-DAP caused responses to increase in magnitude. The effect was greater for botulinum toxin type A than for botulinum toxin types B and E. These results confirm and extend earlier work in which electrophysiological studies showed that aminopyridines were more effective in promoting acetylcholine release from tissues poisoned with toxin type A than from tissues previously poisoned with toxin type B (12) or type F (5).

The data suggest that 3,4-DAP or a drug similar to it might be useful in the treatment of botulism caused by type A toxin. As indicated by the discussion above, the drug might slow the onset and diminish the ultimate severity of the disease. Although additional experiments are needed, the available

findings suggest that 3,4-DAP may increase the lethal dose of botulinum toxin type A (7).

4b. Results and Conclusions from Antibody Experiments

The work is proceeding according to the sequence of steps enumerated above. This is an extremely time-consuming project in protein chemistry. Furthermore, the work has been temporarily delayed due to an equipment alteration. An on-line amino acid analyzer has been purchased to interface with the gas phase sequenator. The company that made the instruments (Applied Biosystems) has asked that the two be returned to the manufacturer for an internal modification that will update the apparatus. The instruments are supposed to be returned on or about November 1, 1986.

During the interim when protein chemistry work is delayed, antibody studies are continuing. In the course of this work a most unusual discovery has been made. The background and the results from the work are summarized here.

In addition to the seven neurotoxins, Clostridium botulinum produces an unusual toxin (C_2) that is composed of two components and which the author calls a binary toxin. The precise mechanism of action of the toxin has not been established, but the following general scheme seems to apply. The heavy chain is a polypeptide that facilitates the binding of the toxin to vulnerable cells. The light chain is an enzyme that possesses ADP-ribosylating activity. The intracellular substrate for the toxin may be actin.

As part of the work on polyclonal and monoclonal antibodies, the light chain of the binary toxin was administered to mice. Surprisingly, it was found that the light chain was not immunogenic. This was an unexpected finding, because the polypeptide has a molecular weight of 50,000 daltons. It is hard to believe that a protein this size lacks antigenic sites.

Several experiments were done to pursue this observation, the following of which was revealing. Groups of mice were injected with the molecule: i.) as the native light chain, ii.) as the toxoided light chain, and iii.) as a combination of the two. The expectations set on the experiment were these. If all three groups failed to produce antibody, the light chain would tentatively be viewed as lacking epitopes. If groups i. and iii. failed to produce antibody, the native light chain would be viewed as an immunosuppressive agent. If only group i. failed to produce antibody, the light chain would be viewed as an agent that impaired only those components of the immune response that interacted with the chain.

It is the latter result that was obtained. This showed that the light chain did have epitopes and it was not a general immunosuppresant. Instead, it appears to possess a property that impairs only that portion of the immune system that comes into contact with the molecule. One way to envision the process is as follows. The light chain is endocytosed as an initial step in the immune mechanism. The endocytosing cell has a substrate (actin?) that is vulnerable to ADP-ribosylation. When the substrate is enzymatically inactivated, the sequence of events in

the immune mechanism is halted. Thus, no endogenous antibody is formed.

There are of course other ways to interpret the data. However, most interpretations share these three properties. Firstly, they suggest that the native light chains of the botulinum neurotoxins should be tested for immunizing ability. This would allow a comparison of the known catalytic activity of the binary toxin with the assumed catalytic activity of the neurotoxins. Secondly, other ADP-ribosylating toxins with light chains should be compared with the binary toxin. This would indicate whether the antibody outcome hinges mainly on the nature of the enzyme or mainly on the nature of the substrate. And thirdly, the light chain of the binary toxin should be tested as a selective, chimeric immunosuppresant. For example, the light chain covalently linked to an antigen for which there are existing antibodies may cause the titer of these antibodies to decrease. All of these are being pursued.

5. Plans for Coming Year

The Principal Investigator will continue the research plan exactly as described in the research proposal. The only change is one that applies to funding, and it falls within accepted guidelines. The expense of preparing monoclonal antibodies is substantial, and it was not anticipated in the original proposal. To offset this expense, the Principal Investigator is lowering his percent time and effort from 40% to 30%. The additional monies will be used to support the monoclonal work. The

reallocation of salary money falls well below the 25% change in any category that is permitted by DOD guidelines.

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